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(54) Chondroitinase compositions

(57) This invention provides pharmaceutical compositions containing chondroitinase wherein a decrease in enzyme activity is very little even after long-term storage, and the pharmaceutical compositions are characterized in that they contain chondroitinase and a pharmaceutical carrier, and that an amount of reducing impurities per 1 g of said pharmaceutical carrier is 0.4 mL or less as a titer by a titration method with 0.01 N of ammonium ceric nitrate.

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(derived from Flavobacterium sp. Hp102; H. Miyazono, H. Kikuchi, K. Yoshida, K. Morikawa, K. Tokuyasu, Seikagaku, 61, 1023 (1989)), chondroitinase B (derived from Flavobacterium heparinum; Y. M. Michelacci, C. P. Dietrich, Biochem. Biophys. Res. Commun., 56, 973 (1974), Y. M. Michelacci, C. P. Dietrich, Biochem. J., 151, 121 (1975), K. Maeyama, A. Tawada, A. Ueno, K. Yoshida, Seikagaku, 57, 1189 (1985)), chondroitinase C (derived from Flavobacterium sp. Hp102; H. Miyazono, H. Kikuchi, K. Yoshida, K. Morikawa, K. Tokuyasu, Seikagaku, 61, 1023 (1989)) and the like are known, and any of these chondroitinase may be used.

Also, chondroitinase having the following physical and chemical properties may be used (see, Seikagaku, 67, 737 (1995)):

① Action:

Hydrolyze a N-acetylhexosamnide linkage in glycosaminoglycan. Action on chondroitin sulfate results in the production of saturated chondroitin sulfate oligosaccharides having 12-16 sugars mainly.

② Substrate specificity:

At pH 5, act on chondroitin sulfate, but not on hyaluronic acid, keratan sulfate, heparan sulfate and heparin.
At pH 3.5, act on chondroitin sulfate and hyaluronic acid.

③ Optimum pH for reaction:

Near pH 5 (substrate: chondroitin sulfate derived from shark cartilage (average molecular weight 44000), buffer: 50 mM citric acid-Na_2HPO_4 buffer containing 0.15M NaCl, temperature: 37°C)

④ Isoelectric point:

Near pH 5

⑤ Molecular weight:

Approximately 36 kDa, in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using a gel wherein polyacrylamide is copolymerized with chondroitin sulfate chains having allyl groups at their ends (zymography; Anal. Biochem. 225, 333-340 (1995)) .

⑥ Endo-type enzyme

⑦ Derived from human.

This enzyme can be obtained, for example, from the surrounding tissue of human gastric cancer by a conventional extraction or purification method for enzymes. More specifically for the extraction method, extraction by a cell disruption-extraction method such as cutting into small pieces with scissors etc., homogenization, sonication, osmotic shock, freeze-thawing and the like, extraction with surfactants, and combinations thereof can be mentioned. Particularly preferred is a method wherein tissue is cut into small pieces with scissors. Also, more specifically for the purification methods, for example, salting out with ammonium sulfate and sodium sulfate etc., centrifugation, dialysis, ultrafiltration, adsorption chromatography, ion-exchange chromatography, hydrophobic chromatography, reversed phase chromatography, gel filtration, gel permeation chromatography, affinity chromatography, electrophoresis, zymography and the like, and combinations thereof can be mentioned, zymography (Anal. Biochem. 225, 333-340 (1995)) being particularly preferred.

In addition, this enzyme can be purified by chromatography with a carrier having heparin as a ligand, since it can be adsorbed to Heparin-Sepharose (Pharmacia).

Furthermore, chondroitinase having the above physical and chemical properties can be also obtained by cloning a gene of this enzyme according to known per se methods, and transfecting it into an appropriate host, followed by th expression. For example, this enzyme may be obtained by the isolation of DNA which codes for this enzyme from human DNA library using a specific chondroitin sulfate degradation activity of said enzyme as an indicator, the insertion into a vector by means of recombinant DNA techniques, the transfection into host cells, and the expression. Alternatively, cloning may be carried out by generating an specific antibody to this enzyme and using said antibody. In addition, cloning may be carried out by the determination of the N-terminal amino acid sequence of this enzyme and the use of DNA or oligonucleotide having a nucleotide sequence deduced from this sequence as a probe. The expressed enzyme

8.0 and 37°C. By using chondroitinase with enzyme specific activity of 300 U/mg protein or more, when administered in vivo as a pharmaceutical product for injection, it can adequately degrade proteoglycan at the target site (e.g. intervertebral disc of mammals, preferably humans, with herniation) without affecting the surrounding tissue, thus being able to afford a pharmaceutical product with high safety and high efficiency.

Most preferable chondroitinase used in pharmaceutical compositions of the invention is purified chondroitinase ABC having the following properties.

(i) The molecular weight is approximately 100,000 dalton in the measurements by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (under both reducing and non-reducing conditions) and by gel filtration.
(ii) The isoelectric point is about pH 8.2 and about pH 8.5.
(iii) The optimum pH is 8.0-8.2 (substrate: chondroitin sulfate C, buffer: Tris-HCl buffer), and the optimum temperature is 37°C.
(iv) The activity is inhibited by Zn^{2+}, Ni^{2+}, Fe^{3+} and Cu^{2+}.
(v) The N-terminal amino acid is alanine, and the C-terminal amino acid is proline.
(vi) A single band appears in SDS-PAGE, and a single peak is also obtained in high performance liquid chromatography (gel filtration and cation-exchange).
(vii) No endotoxin is contained substantially, and a nucleic acid content and a protease content are below the detection limit.
(viii) Can be crystalized.
(ix) The specific activity is 300 U/mg or more.

Such chondroitinase ABC may be obtained, for example, according to the methods described in U.S. Patent No. 5,496,718 and EP-A-576,294.

Pharmaceutical carrier:

As pharmaceutical carriers in pharmaceutical compositions of the invention, used are those wherein an amount of reducing impurities per 1 g of said pharmaceutical carrier is 0.4 mL or less, preferably 0.36 mL or less, as a titer by a titration method with 0.01 N ammonium ceric nitrate. In addition, those wherein a peroxide content is 20 ppm or less, particularly 18.5 ppm or less are more preferred. Furthermore, pharmaceutical carriers used in pharmaceutical compositions of the invention are preferably those which are treated with activated carbon.

Herein, the term "a titration method with 0.01 N ammonium ceric nitrate" refers to a method wherein 2 g of a pharmaceutical carrier is dissolved in 25 mL of warm water, 25 mL of dilute sulfuric acid and 0.1 mL of ferroin (tris(1,10-phenanthroline) iron (II) complex: $[Fe(C_{12}H_8N_2)_3]^{2+}$) are added, and the mixture is titrated with 0.01 N ammonium ceric nitrate until the color is changed to greenish-blue from red and held for 30 seconds. By measuring the volume of 0.01 N ammonium ceric nitrate used for the titration, and then by converting it to a titer per 1 g of a pharmaceutical carrier, an amount of reducing impurities can be obtained.

As examples of pharmaceutical carriers, additives, which are generally used for pharmaceutical products, such as conventional diluents, binders, lubricants, coloring agents, disintegrating agents, buffer agents, isotonizing agents, preservants, anesthetics and the like are given.

Preferably, pharmaceutical carriers used in pharmaceutical compositions of the invention are purified to the extent that they can be used as a pharmaceutical product, and do not substantially contain pharmaceutically unacceptable substance. More specifically for such pharmaceutical carriers, for example, dextran, sucrose, lactose, maltose, xylose, trehalose, mannitol, xylitol, sorbitol, inositol, serum albumin, gelatin, creatinine, polyethylene glycol, non-ionic surfactants (e.g. polyoxyethylene sorbitan fatty acid esters, polyoxyethylene hardened castor oil, sucrose fatty acid esters, polyoxyethylene polyoxypropylene glycol) and the like can be mentioned. As examples of the above polyoxyethylene sorbitan fatty acid esters (polysorbates), polyoxyethylene sorbitan (the polymerization degree of ethylene oxide: about 20) monolaurate, monopalmitate, monooleate, monostearate, trioleate and the like can be given. As commercial products, Polysorbate 80 (polyoxyethylene sorbitan monooleate (20 E.O.)), Polysorbate 60 (polyoxyethylene sorbitan monostearate (20 E.O.)), Polysorbate 40 (polyoxyethylene sorbitan monopalmitate (20 E.O.)), Polysorbate 20 (polyoxyethylene sorbitan monolaurate (20 E.O.)), Polysorbate 21, 81, 65, 85 and the like can be named (Here, 20 E.O. means that the polymerization degree of ethylene oxide in polyoxyethylene moiety is about 20). As examples of polyoxyethylene hardened caster oil, commercially available HCO-10^R, HCO-50^R, HCO-60^R (Nikko Chemicals Co., Ltd.) and the like can be given. As sucrose fatty acid esters, commercially available DK ester F-160^R (Dai-ichi Kogyo Seiyaku Co., Ltd.), Ryoto sugar ester^R (Mitsubishi Kagaku Foods) and the like can be mentioned. As polyoxyethylene polyoxypropylene glycol (poloxamer), commercially available Pluronic F-68^R (Asahi Denka Kogyo K.K.) and the like can be given.

Buffer agents can be any of those which are physiologically acceptable, and are not particularly limited. For example, buffer agents containing one or more of hydrochloric acid, sodium hydrooxide, sodium carbonate, sodium hydro-

desirably used in pharmaceutical compositions of the invention can be lowered, for example, by treating a pharmaceutical carrier with activated carbon in accordance with conventional methods. The amount of peroxide can be also lowered by heat-treatment of a pharmaceutical carrier.

The mixing ratio of chondroitinase and a pharmaceutical carrier in pharmaceutical compositions of the invention is not particularly limited, and can be suitably determined by those skilled in the art depending on the amount for administration, the form of pharmaceutical compositions of the invention and the like. For example, when pharmaceutical compositions of the invention is provided (stored) in the form of freeze-dried preparations, a preferred content of chondroitinase in pharmaceutical compositions of the invention is such that the shape of freeze-dried cake can be kept.

Pharmaceutical compositions of the invention can be prepared by known per se methods using chondroitinase and a pharmaceutical carrier as described above. Pharmaceutical compositions of the invention may be also in either solution, frozen, or dried form.

Pharmaceutical compositions of the invention, when provided in solution form, can be prepared, for example, by making a pH-adjusted buffer solution, and adding the abovementioned chondroitinase and a pharmaceutical carrier to give a solution containing 5 U/mL or more, more preferably 10-400 U/mL chondroitinase, and if necessary, followed by sterilization by filtration.

Pharmaceutical compositions of the invention, when provided in frozen form, can be prepared, for example, by freezing pharmaceutical compositions of the invention in solution form as described above, for example, at -80 - -18°C.

Pharmaceutical compositions of the invention, when provided in dried form, can be prepared, for example, by drying pharmaceutical compositions of the invention in solution form as described above under non-heating conditions such as freeze-drying and the like.

Among these, preferred form of pharmaceutical compositions of the invention is dried from, and more preferred is freeze-dried form, that is, freeze-dried preparations.

It is desirable to adjust the pH of pharmaceutical compositions of the invention usually to pH 5-9, and preferably to pH 6-8, in solution form (in case pharmaceutical compositions of the invention are in frozen form, solution form before freezing and after thawing, and in case pharmaceutical compositions of the invention are freeze-dried compositions, solution form before freeze-drying and after reconstituted by the addition of a solvent). For this purpose, a buffer agent which is capable of stabilizing the pH in said pH region is usually mixed into pharmaceutical compositions of the invention. As said buffer agents, any physiologically acceptable buffer agents may be used, without particular limitation, and for example, hydrochloric acid, sodium hydrooxide, sodium carbonate, sodium hydrogencarbonate, phosphoric acid, potassium dihydrogenphosphate, dipotassium hydrogenphosphate, sodium dihydrogenphosphate, disodium hydrogenphosphate, aminoacetic acid, sodium benzoate, citric acid, sodium citrate, acetic acid, sodium acetate, tartaric acid, sodium tartarate, lactic acid, sodium lactate, ethanolamine, arginine, ethylenediamine, or mixtures thereof can be mentioned. Phosphate buffer solution (agent) is particularly preferred. By means of these buffer agents, the pH of pharmaceutical compositions of the invention can be adjusted to and stabilized at the pH range of pH 5-9, and preferably pH 6-8, in solution form. When the pH is lower than 5 or higher than 9, chondroitinase may be inactivated, or insoluble matter may be produced in solution form. The concentration of a buffer agent in pharmaceutical compositions of the invention may be made to 1 mM or more, and preferably 10-50 mM. Pharmaceutical compositions of the invention may include, in addition to a buffer agent, components required for isotonization (salts such as sodium chloride, etc., sugars, and the like), preservants, anesthetics and the like.

The pharmaceutical compositions of the invention may be used as final dosage form to be administered as a pharmaceutical product as such, or as a material for final dosage form of other pharmaceutical products, for example, solution preparations, freeze-dried preparations and the like.

The pharmaceutical compositions of the invention are mainly used as injection preparations containing chondroitinase as the active ingredient. When pharmaceutical compositions of the invention are provided as injection preparations in solution form, the pharmaceutical compositions of the invention in solution form, prepared by th abovementioned method, may be filled and sealed in appropriate containers such as ampules, vials, syringes for injection and the like, distributed as such or stored, and served as injection preparations for administration.

When pharmaceutical compositions of the invention are provided as injection preparations in frozen form, the pharmaceutical compositions of the invention in frozen form, prepared by the abovementioned method, may be filled and sealed in appropriate containers such as ampules, vials, syringes for injection and the like, distributed as such or stored, and melted before administration to serve as injection preparations.

Preferably, the distribution and storage are carried out at the temperature of -80 - -25°C.

When pharmaceutical compositions of the invention are provided as injection preparations in dried form, the pharmaceutical compositions of the invention in dried form, prepared by the abovementioned method, may be filled and sealed in appropriate containers such as ampules, vials, syringes for injection and the like, distributed as such or stored, reconstituted with water for injection, physiological saline, aqueous glucose solution or aqueous sorbitol solution, and the like before administration to serve as injection preparations. The pharmaceutical compositions of the invention in dried form may be provided together with a solvent for reconstitution.

The enzymatic activity of chondroitinase ABC was determined by the following method.

Using 1.2 mg of chondroitin sulfate C as a substrate, to a 50 mM Tris-HCl buffer solution (pH 8) containing 50 mM sodium acetate, and 10 μg of casein, an enzyme sample (chondroitinase ABC) was added, the reaction carried out at 37°C for 10 minutes, then terminated by the addition of 0.05 M hydrochloric acid with pH 1.8, and the absorbance in the ultraviolet region (at 232 nm) was measured. On the other hand, as a control, a heat-denatured enzyme sample was hold in a substrate solution having the abovementioned composition, similar procedures performed, and the absorbance at 232 nm was measured. The amount of unsaturated disaccharide produced by the action of chondroitinase ABC was calculated from an increase in the absorbance compared to control. One unit (U) of an enzyme was defined as an amount of the enzyme required to catalize a reaction wherein 1 micromole of unsaturated disaccharide is released for 1 minutes under the abovementioned reaction condition, considering the millimolar absorption coefficient of 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose as 5.5.

As a result, this chondroitinase ABC was verified to have specific activity of 300 U/mg protein or more.

Example 1: Preparation of pharmaceutical compositions of the invention consisting of chondroitinase ABC and Polysorbate 80

Polysorbate 80 was obtained from several manufactures, and an amount of reducing impurities and a concentration of peroxide in 1 g of Polysorbate 80 were determined in accordance with the abovementioned methods.

Then, sodium chloride (0.75%), the above highly purified chondroitinase ABC (specific activity 350 U/mg; final concentration 25 U/mL) and Polysorbate 80 (0.15% (w/w)) were dissolved in a 20 mM phosphate buffer solution (pH 7) to prepare a composition (solution). The solution was sterilized by filtration using a membrane filter (pore size 0.22 μm; Millex GV, MILLIPORE), and every 2 mL was filled into sterilized glass ampules, which were subsequently sealed.

The sealed glass ampules were stored for one month at 25°C, and then evaluation was carried out by the following methods.

Evaluation methods:

(1) Appearance: Visual observation was made at a position of luminous intensity of about 1000 luxes, right under an incandescent electric bulb with a white paper and a black paper on the background, .

(2) Measurement of enzyme activity of chondroitinase ABC: Using a solution before storage or a solution after storage as "an enzyme sample" in the above-mentioned method for the measurement of enzyme activity, the measurement was carried out.

The percentage of enzyme activity of a solution after storage (Post solution) to that of a solution before storage (Pre solution) was calculated with the following equation, and variations in enzyme activity by one month storage at 25°C were compared.

[Enzyme activity of Post solution / Enzyme activity of Pre solution] x 100 (%)

Results are shown in Table 1 as "stability", along with the results from the measurements of a amount of reducing impulities and a concentration of peroxide in 1 g of a pharmaceutical carrier (Polysorbate 80). Here, "N/T" in Table 1 means not being tested.

Table 1

Pharmaceutical carrier (Polysorbate 80)	Amount of reducing impurities (mL) (titer per 1 g)	Concentration of peroxide (ppm)	Stability (%)
A	0.27	N/T	87
B	0.36	16.0	85
C	0.33	N/T	84
D	0.28	N/T	83
E	0.08	N/T	78
F	0.29	N/T	75

Example 3: Preparation of pharmaceutical compositions of the invention (freeze-dried preparations) consisting of chondroitinase ABC and polyethylene glycol

(1) Polyethylene glycol used in this example

Polyethylene glycol 4000 (PEG 4000, average molecular weight 2600-3800; Wako Pure Chemical Industries, Ltd., first grade, Lot. No. CAE 0369) was dissolved in endotoxin-free distilled water (water for injection), this aqueous solution treated with activated carbon, and an amount of reducing impurities and a content of peroxide were measured according to the abovementioned methods. As a result, the amount of reducing impurities in a 4 % (w/v) polyethylene glycol solution was 0 mL as a titer, and the content of peroxide in a 6.66 % (w/v) polyethylene glycol solution was below the detection limit (below 0.0017 % (w/v).

(2) Test method

(Stability test)

Chondroitinase ABC (final concentration 40 U/mL) and polyethylene glycol 4000 (final concentration 1 % (w/w)) were dissolved in a 10 mM phosphate buffer solution (pH 7), and every 0.5 mL was filled into vials (giving 20 U/vial as chondroitinase ABC), which were subsequently freeze-dried. Freeze-drying is performed as follows: cool-freezing from room temperature to -45°C, primary drying for 12 hours under reduced pressure (60 mTorr), heat-up to 25°C (12 hours), and secondary drying for 10 hours at 25°C. After the drying, pressure was restored with nitrogen gas, and vials were capped.

Then, the percentage of "the enzyme activity of a reconstituted solution of a freeze-dried composition after stored for 30 days under 40°C" to "the enzyme activity of a solution before freeze-drying" was obtained in accordance with the following procedure. Enzyme activity of chondroitinase after freeze-drying (hereinbelow, "enzyme activity of chondroitinase" may be simply called as "enzyme activity"), appearance of cake, re-solubility and a water content were evaluated.

Enzyme activity was determined using a solution before freeze-drying or a reconstituted solution with physiological saline after freeze-drying as an enzyme sample, in accordance with the abovementioned method for the measurement of enzyme activity.

The percentage of the enzyme activity of a reconstituted solution after freeze-drying (Post-FD solution) to the enzyme activity of a solution before freeze-drying (Pre-FD solution) was calculated with the following equation, and the variations in enzyme activity at the time of freeze-drying and those during subsequent storage were compared.

[Enzyme activity of Post-FD / Enzyme activity of Pre-FD] x 100 %

Appearance of cake was visually observed. When the appearance of dried cake after freeze-drying was in good tablet form, it was identified as "good", and when the appearance of dried cake after freeze-drying was not in good tablet form, it was identified as "poor".

Re-solubility was determined after freeze-drying by observing the solubility when reconstituted with 2 mL of physiological saline and the appearance of the solution after reconstituted. More specifically, reconstitution was first confirmed within one minute after the addition of physiological saline. When insoluble particulate matter was found in the dissolved solution by visual observation, the result was identified as "+", and when the solution was clear and no particulate matter was observed, the result was identified as "-". The content of water was measured according to the abovementioned TG Method.

(3) Results

The results of stability test with freeze-dried preparations consisting of a combination of chondroitinase ABC and polyethylene glycol 4000 showed that in freeze-dried preparations consisting of a combination of chondroitinase ABC and polyethylene glycol 4000, the enzyme activity decreased little even after storage for 30 days under 40°C (the percentage of enzyme activity of Post-FD to that of Pre-FD is 76.1 %) and was retained very stably.

Also, a water content in the freeze-dried preparations was as low as 3 % (w/w) or less, appearance of cake was "good", and re-solubility was "-".

(4) Results

The results of stability test (storage at 40°C, for 30 days) with freeze-dried preparations consisting of a combination of chondroitinase ABC, sucrose and polyethylene glycol 4000 showed that in freeze-dried preparations consisting of a combination of chondroitinase ABC, sucrose and polyethylene glycol 4000, the enzyme activity decreased little even after storage for 30 days at 40°C (the percentage of enzyme activity in Post-FD to that in Pre-FD was 90.9 %) and was retained very stably.

Also, a water content in the freeze-dried preparations was as low as 1.5 % (w/w) or less, appearance of cake was "good", and re-solubility was "-".

As mentioned above, pharmaceutical compositions of the invention consist of chondroitinase, preferably chondroitinase with high specific activity and high purity, and a pharmaceutical carrier wherein an amount of reducing impurities is 0.4 mL or less per 1 g as a titer by a titration method with 0.01 N ammonium ceric nitrate and desirably a concentration of peroxide is 20 ppm or less.

By selecting such constitution for compositions, the present invention can provide pharmaceutical compositions wherein a decrease in enzyme activity of chondroitinase is very little after long-term storage. Furthermore, by using pharmaceutical compositions of the invention, the invention can provides pharmaceutical products, particularly agents for the treatment of disc herniation, which are safe, effective, easy to handle and can be stored for long time.

Claims

1. A pharmaceutical composition, characterized in that it contain chondroitinase and a pharmaceutical carrier, and that an amount of reducing impurities contained in 1 g of said pharmaceutical carrier is 0.4 mL or less as a titer by a titration method with 0.01 N ammonium ceric nitrate.

2. The pharmaceutical composition according to claim 1, wherein a peroxide content in the pharmaceutical carrier is 20 ppm or less.

3. The pharmaceutical composition according to claim 1, wherein an amount of reducing impurities contained in 1 g of the pharmaceutical carrier is 0.36 mL or less as a titer by a titration method with 0.01 N ammonium ceric nitrate.

4. The pharmaceutical composition according to claim 1, wherein the pharmaceutical carrier is treated with activated carbon.

5. The pharmaceutical composition according to claim 1, wherein the pharmaceutical carrier is sucrose and/or polyethylene glycol.

6. The pharmaceutical composition according to claim 1, wherein the pharmaceutical carrier is polyethylene glycol.

7. The pharmaceutical composition according to claim 1, wherein the pharmaceutical carrier is a mixture of sucrose and polyethylene glycol.

8. The pharmaceutical composition according to claim 5, wherein the mixed ratio of polyethylene glycol and sucrose is 0/1-10/1 by weight ratio of polyethylene glycol/sucrose.

9. The pharmaceutical composition according to claim 7, wherein the mixed ratio of polyethylene glycol and sucrose is 2/1 by the weight ratio of polyethylene glycol/sucrose.

10. The pharmaceutical composition according to claim 5 or 6, wherein polyethylene glycol has an average molecular weight of 3000-4000.

11. The pharmaceutical composition according to claim 1, wherein the pharmaceutical carrier is polyoxyethylene sorbitan fatty acid ester.

12. The pharmaceutical composition according to claim 1, wherein chondroitinase is chondroitinase ABC.

13. The pharmaceutical composition according to claim 1, wherein specific activity of chondroitinase ABC is 300 U/mg protein or more.

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PARTIAL EUROPEAN SEARCH REPORT

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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	TAKAHASHI T ET AL: "Treatment of Canine Intervertebral Disc Displacement with Chondroitinase ABC" SPINE,US,PHILADELPHIA, PA, vol. 22, no. 13, 1 July 1997 (1997-07-01), pages 1435-1439, XP002090506 * see abstract and page 1436 left col. * ---	1-4, 15-17	
X	PATENT ABSTRACTS OF JAPAN vol. 017, no. 171 (C-1044), 2 April 1993 (1993-04-02) & JP 04 330280 A (SEIKAGAKU KOGYO CO LTD), 18 November 1992 (1992-11-18) * abstract * ---	1-5,8, 15-17	
Y	WO 93 00807 A (CRYOLIFE INC) 21 January 1993 (1993-01-21) * see claims 1-3 and 21 * -----	1-17	TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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